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Rapid synthesis of long-chain deoxyribooligonucleotides by the N-methylimidazolide phosphotriester method

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ABSTRACT

A modified phosphotriester method has been employed for the efficient chemical synthesis of long-chain deoxyribooligonucleotides. During the course of this work, a general and rapid procedure was developed for the preparation of 24-62-mers in solution. Preparative reversed phase column chromatography on silanized silica gel was used to purify triester intermediates starting from 10-mers. The rapid synthesis of 32-mer and 42-mer on glass and silica gel supports using suitably protected 2-8-mer blocks as coupling units has been also accomplished. In particular, a convenient procedure for the solid-phase synthesis of oligonucleotide blocks bearing 3'-terminal phosphodiester groups is described.

INTRODUCTION

Recent developments in recombinant DNA technology have been greatly aided by the availability of synthetic DNA fragments with defined sequences. In the modern oligonucleotide synthesis, the phosphotriester and the phosphite triester approaches are the most widely used. These approaches are developed sufficiently to allow rapid preparation of deoxyribooligonucleotides ranging in sizes from 8-20-mers, which are in great demand for various molecular-biology studies [1,2]. However, certain goals need longer synthetic single-stranded polynucleotides, and the initial steps towards the chemical synthesis of 30-50-mers have been recently done in number of laboratories [3-5]. For example, the availability of such DNA fragments permits acceleration of chemical-enzymatic synthesis of artificial genetic structures [6], it facilitates the realization of complex cases of site-specific mutagenesis of cloned DNA fragments [7] and reveals new possibilities for physical studies of nucleic acids.

Earlier, we described the rapid variant of the phosphotriester approach based on the use of highly effective coupling reagents, arylsulfonyl chlorides in the presence of N-methylimidazole (MeIm), for phosphotriester bond formation [5,8]. These reagents allow to perform internucleotide condensations not only in pyridine, which is a traditional solvent for the oligonucleotide synthesis, but also in other organic solvents, such as acetonitrile, methylene chloride, dioxane, chloroform, etc. Furthermore, this method completely excludes the use of tetrazole and triazole derivatives as condensing reagents for internucleotide bond formation and phosphorylating agents in preparation of suitably protected mononucleotides. The effectiveness of this rapid phosphotriester method in homogeneous solution and on solid phase has been demonstrated by the successful synthesis of more than 60 deoxyribooligonucleotides of 12-18 nucleotides in length and several longer DNA fragments (20-32-mers)[5,8-10].

In this communication, we describe some improvements in N-methylimidazolide method and its application to the efficient synthesis of long-chain oligonucleotides up to 62-mer. The sequences of these compounds, representing the fragments of synthetic genes for bacteriorhodopsin (I - V) and human preproinsulin (VI-VIII), are shown in Table 1.

RESULTS AND DISCUSSION

Polynucleotide synthesis in solution

Despite recent achievements in the oligonucleotide synthesis on polymer supports, the synthesis in homogeneous solution still had not lost its value, especially when relatively large amounts of polynucleotides (hundreds or thousands of optical units) are needed. Moreover, compared to solid-phase synthesis, the solution variant has an advantage in purification of intermediates, that results in higher purity of the final product and, hence, in simplification of its isolation after the removal of the protecting groups.

The N-methylimidazolide phosphotriester method speeds up considerably the oligonucleotide synthesis in solution and, in conjunction with the improved methods of purifying intermediates,

TABLE 1. Oligonucleotides prepared by solution (S) and solid-phase (P) chemistry

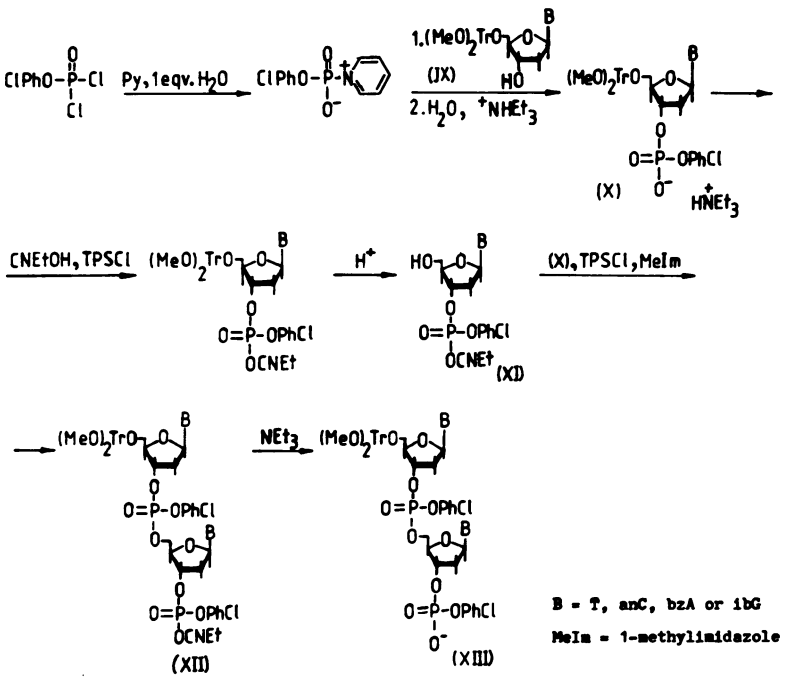
Compound	Sequence	Method of preparation	Synthesis scheme[a]	Yield, % Overall	Yield, % Isolated[c]
(I)	d(CCTGATCCTCCTTCGTTCTCGTGC)	S	2+2+4+8+8	24	7.6
(II)	d(CTTAACCGAAGATCGCACGAGAAC)	S	2+2+4+8+8	21	8.4
(III)	d(TCGAAACTCTTCTGTTCATCGTTCTTGATGTT)	S	2+2+4+8+8+8	19	5.0
(IV)	d(CCGAAACCCACTTTAGCAGAAACATCAAGAAC)	S	2+4+4+4+8+10	17	6.0
(V)	d(CCGAAACCCACATTAGCAGAAACATCAAGAAC)	P	1+1+4x7+2	23	5.8
(VI)	d(GCTGCAGCTGGATCAGGACCCCACAGAGCCAGC-AGAGCCAGC)	P	1+2+6+4+6+4+6+5+8	10	2.1
(VII)	d(GAATTCATGGCCCTCTGGATGCGCCTCCTGCCT-CTGCTGGCTCTGCT)	S	2+2+2+4+10+8+8+11	7.9	1.0
(VIII)	d(GAATTCATAAGGTAATTCAAAATGGCCCTCTGGA-TGCGCCTCCTGCCTCTGCTGGCTCTGCT)	S	2+2+2+4+10+8+8+8+10+8	2.0	0.5

[a] The syntheses were carried out in the 3'-5' direction.

[b] Yields are based on 3'-terminal dinucleoside monophosphate in the solution synthesis and on the first 5'-dimethoxytritylnucleoside attached to resin in the solid-phase synthesis.

[c] After HPLC on Zorbax C-8 column.

Nucleic Acids Research



Scheme 1

makes possible the rapid synthesis of long-chain oligomers on 10-100 μmole scale. The utility of this methodology is exemplified in this paper by the synthesis of the six 24-62 nucleotide-long DNA fragments in good overall yields.

In the syntheses of compounds (I - IV), (VII) and (VIII) (Table 1), the polynucleotide chains were elongated in the 3'-5' direction by a successive addition of di-, tetra-, octa- and longer oligonucleotide blocks to the 3'-terminal dinucleoside monophosphate. Preparation of appropriately protected monomers, 5'-O-dimethoxytrityl-2'-deoxynucleoside-3'-O-chlorophenyl phosphates (X) and deoxynucleoside-3'-O-chlorophenyl-β-cyanoethyl phosphates (XI), and dinucleotides (XII, XIII) was accomplished with the use of simplified procedures, which were described by us previously [5,11] (Scheme 1). During the syntheses, 1.2-1.5-fold molar excess of 3'-P-component over 5'-OH-component, 2-3-fold excess of arylsulfonyl chloride and 4-6-fold excess of MeIm relative to P-component were usually used in condensation

reactions. Acetonitrile was used as a solvent in preparation of short oligomers. Due to low solubility of longer oligonucleotides in pure acetonitrile, the synthesis of compounds starting from 6-8-mers was carried out in pyridine or methylene chloride (or in the mixtures of these solvents with acetonitrile)[5]. As it was expected, the rates of internucleotide condensations decreased with the increasing molecular weights of OH- and P-components involved in the reaction. This was caused by reduction of the upper limits of their possible concentrations in the reaction mixture and by lower mobility of these large molecules in solution. Thus, the optimal coupling times were found to be 5-10 min for 2-8-mers, 10-20 min for 10-20-mers and 20-40 min for 24-60-mers. The yields on the steps of 4-16-mers were in the range of 75-90%, whereas in the cases of 32-62-mers they dropped to 40-50%. It should be noted that increasing the molar excess of P-component over OH-component up to 3-5-fold in the case of 30-60-mers led to a slight increase in the rate of phosphotriester bond formation and to higher yields of the desired product.

The removal of 5'-O-dimethoxytrityl protecting groups from the fully blocked oligonucleotides before the next condensation step was accomplished with the use of 2% toluenesulfonic acid (TSA) solution in chloroform-methanol mixture (7:3) at 0°C. Along with this conventional procedure, the treatment of oligonucleotides with 2% solution of trifluoroacetic acid (TFA) in chloroform (or methylene chloride) containing 5-10% methanol at room temperature was used. Comparative investigation of detritylation and depurination rates under the action of these two reagents revealed that detritylation rate was nearly the same for TSA and TFA in chloroform-methanol mixture, while the depurination rate was considerably slower in the case of TFA[9]. The deprotection with 2% TFA in this solvent was reproducibly complete within 1-2 min and very little depurination (less than 0.05% per min) was observed. This reagent was usually applied for detritylation of long-chain oligonucleotides when depurination is especially undesirable and hard to control.

The fully protected oligonucleotides were purified at initial stages of the synthesis by short column silica gel chromatogra-

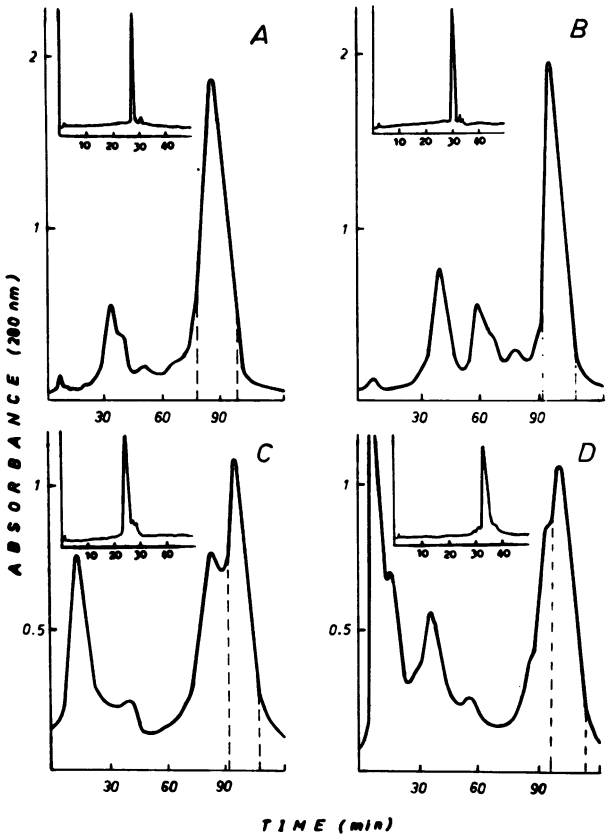


FIGURE 1. Chromatography of the reaction products formed in the syntheses of 24-mer (I)(A), 32-mer (III)(B), 47-mer (VII)(C) and 62-mer (VIII)(D) on Nucleosil 30 C-18 column. The fractions were pooled as shown by vertical dashed lines. Insets: Zorbax C-8 profiles of the purified polynucleotides after the complete deprotection.

phy, whereas 6-10-mers were isolated by HPLC on Lichrosorb Si preparative column using a methanol concentration gradient (2-8%) in chloroform as an eluent. Purification of longer oligonucleotides was performed by means of reversed phase chromatography on alkylated silica gel. Earlier we have shown that the purification of fully protected 8-16-mers can be achieved by the preparative reversed phase chromatography on Nucleosil 30 C_{18} columns in aqueous acetonitrile and dioxane [5,9]. In further examination of the use of this technique, we noticed that it can

be extended to the isolation of longer oligonucleotides up to 60-mers. The chromatography was carried out in a linear gradient of acetonitrile in 0.1 M triethylammonium acetate containing dioxane, or in a gradient of dioxane in the same buffer. Along with the Nucleosil C_{18} column, we used columns packed with RP-2 silica gel, prepared as described by Kalashnikov et al.[12]. Usually, the most delayed peak contained the essentially pure fully protected desired product, and it could be easily identified (Fig. 1).

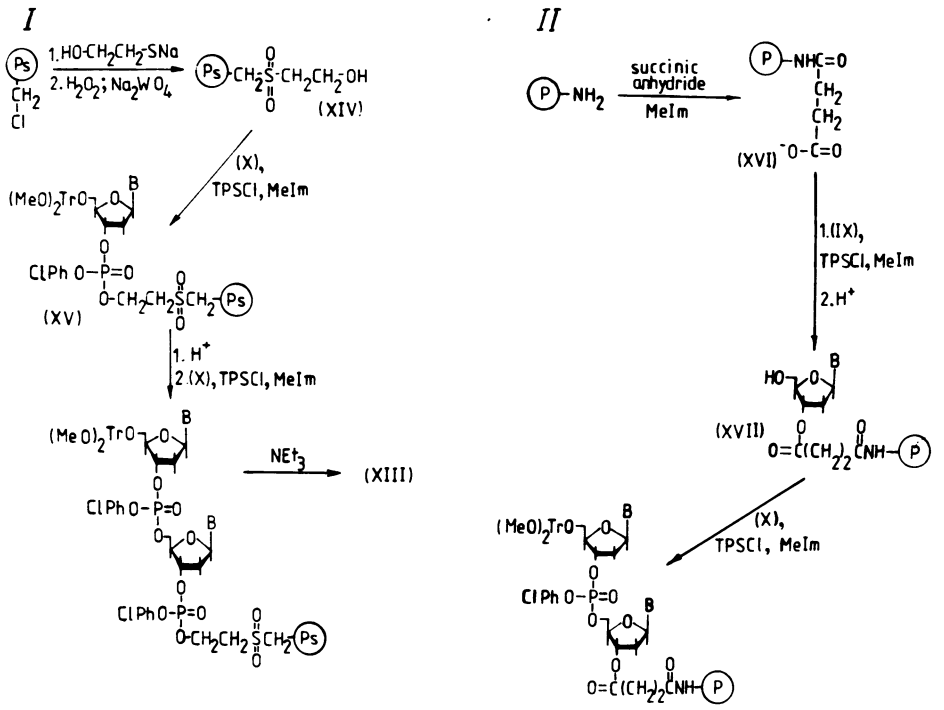
It should be noted that silica gel chromatography, even in its HPLC variant, fails to provide good separation of desired compound from the starting material and side-products on dodeca-tetradecamers level already. This diminishes the yields at subsequent steps of the synthesis and complicates the purification of the final oligonucleotide. The reversed phase chromatography can resolve impurities which can not be removed by silica gel chromatography, namely nonreacted starting OH-component and 5'-sulfonated by-product. Unlike the adsorbtion chromatography, the reversed phase technique can also provide the resolution of compounds with a terminal phosphodiester group.

Solid-phase synthesis of polynucleotides

Earlier we have shown the effectiveness of N-methylimidazolide phosphotriester method in the solid-phase synthesis of 12-16 nucleotide-long oligomers using mono- and dinucleotides as building blocks for chain elongation [5,9]. In order to investigate the feasibility of this method for the synthesis of long-chain oligonucleotides, we have undertaken the synthesis of two DNA 32- and 42-mers, (V) and (VI) (Table 1). In this experiments we have also examined the efficiency of using 4-8-mer P-components in the coupling reactions on polymer supports. The oligonucleotide blocks with 3'-terminal phosphodiester group, which were used as P-components for elongation of polynucleotide chains, were synthesized in solution as described above, or on specially prepared polymer support.

Recently, Balgobin et al.[13] proposed 2-phenylsulfonylethyl group as a phosphate protecting for the blocking of the 3'-terminal phosphodiester to the triester level, and demonstrated its effectiveness in the synthesis of oligonucleotides in solution.

Scheme 2



Considering these data, we used similar group for attachment of the first nucleotide to polymer support and examined this carrier for preparation of oligonucleotides with 3'-terminal phosphodiester group. To prepare the support (Scheme 2, I), the commercially available chloromethyl polystyrene (crosslinked with 1% of divinylbenzene) was derivatized to form a thioethanol polymer, which was converted to sulfonylethanol derivative (XIV) in good yield by oxidation with hydrogen peroxide in the presence of tungstate ions [14]. The polymer obtained was anchored to the 3'-phosphodiester group of the first 5'-dimethoxytrityl deoxynucleotide (X) with the help of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) and MeIm. After capping of unreacted hydroxyl groups on polymer and removal of dimethoxytrityl group from the polymer-bound nucleotide, the oligonucleotide chain

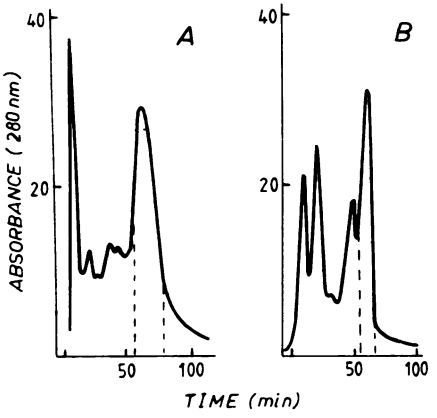


FIGURE 2. Elution profiles on Nucleosil 30 C-18 column using a 45-75% acetonitrile gradient in 0.025 M triethylammonium acetate. The fractions were pooled as shown by vertical dashed lines. (A) d[$(MeO)_2$Tr]anC$_T$anC$_T$anC$_T$bzA-(ClPh); (B) d[$(MeO)_2$Tr]anC$_T$bzA$_T$T$_T$anC--(ClPh). [($_T$) - a protected phosphodiester bond; (ClPh) - p-chlorophenyl].

was elongated in the conditions described by us earlier for the synthesis on polystyrene support [6,9] (Table 2). Synthetic cycles were repeated using different protected nucleotides until the desired sequence was constructed, and the oligonucleotide obtained was cleaved from the support by the action of triethylamine in dioxane for 2-3 hrs at 20°C. After removal from the polymer, the desired oligonucleotide block was isolated by reversed phase chromatography on Nucleosil C_{18} (Fig. 2). This technique was applied to the synthesis of several tetramers on a 50-100 μmole scale, and the three of them, d[$(MeO)_2$Tr]anC$_T$anC$_T$anC$_T$bzA-(ClPh), d[$(MeO)_2$Tr]anC$_T$bzA$_T$T$_T$anC-(ClPh) and d[$(MeO)_2$Tr]-anC$_T$bzA$_T$ibG$_T$anC-(ClPh) were used in the syntheses of long-chain oligonucleotides (V) and (VI). The overall yields of tetramers in these syntheses were estimated to be 60-75% from the total recovered absorbance units of the product based on the first nucleotide. While only a limited number of oligonucleotides has been prepared, the method is clearly promising for further work. It can be also applied to the synthesis of longer oligonucleotides using mono- and dimer building units. The condensing reagents on the base of tetrazole and 3-nitro-1,2,4-triazole can be also used [15].

The polynucleotides (V) and (VI) were obtained with the use of non-swellable supports on the base of porous glass beads and HPLC-grade silica gel, respectively. In order to attach the first nucleoside to the supports, the following two steps pro-

TABLE 2. Reaction cycle for chemical synthesis of oligonucleotides on polymer supports

Step	Reagent or solvent [a]	Time(min)
Polystyrene support		
1	2% TFA in CH_2Cl_2-CH_3CN (15:5,v/v), 3 ml	6
2	CH_2Cl_2 , 10 ml	3
3	Coupling mixture in CH_2Cl_2 [b]	20-30
4	CH_2Cl_2, 10 ml	5
Glass and silica gel supports [c]		
1	2% TFA in CH_3CN-CH_2Cl_2(7:3,v/v)	5-7
2	CH_3CN-CH_2Cl_2(7:3,v/v)	2
3	Coupling mixture in CH_3CN-CH_2Cl_2(7:3,v/v)[b], 0.3 ml	20 (recycle)
4	CH_3CN-CH_2Cl_2 (7:3,v/v)	2
5	dioxane-H_2O-DBU [d](14:5:1,v/v)	3-5
6	CH_3CN-CH_2Cl_2 (7:3,v/v)	2
7	CH_3CN-Ac_2O-MeIm (17:2:1, v/v)	3-5
8	CH_3CN-CH_2Cl_2 (7:3, v/v)	2

[a] Amounts of reagents are given per 100 mg of a support.

[b] The nucleotide component (4-5 equiv.) and MeIm (16-20 equiv.) are dried by coevaporation with pyridine or acetonitrile, then a solution of TPSCl (8-10 equiv.) in dry solvent is added, and the reaction solution is injected into the reaction vessel.

[c] The synthesis was performed in a column connected to a manually operated continuous-flow system with the flow rate of 1-2 ml/min.

[d] DBU = 1,8-diazabicyclo(5.4.0)undec-7-ene(1,5-5).

cedure was applied (Scheme 2, II). Firstly, the aminated support was treated with succinic anhydride in pyridine as it was described [16]. Then, the succinylated polymer obtained was condensed with N-protected 5'-dimethoxytrityl nucleosode using TPSCl and MeIm as condensing agent. It was revealed that the latter reaction is complete in 1 h, and the loadings of nucleosides were found to be in the range of 50-100 μmole per gramm as determined by spectrophotometrical analysis of dimethoxytrityl cation released [9].

After the masking remaining carboxyl groups on polymer sup-

port, the growing polynucleotide chain was elongated by a successive addition of the appropriately protected mono-, di- and longer oligonucleotides containing chlorophenyl protected 3'-phosphates. At each step, the 4-5-fold excess of P-component over the resin capacity was used. The coupling reactions were performed in the presence of 2-fold excess of arylsulfonyl chloride and 4-6-fold excess of MeIm with respect to a P-component during 20-30 min. The manipulations of a complete elongation cycle are listed in Table 2. In both cases, the one-solvent procedure for rapid solid-phase synthesis, developed by us earlier [5], was used.

The removal of dimethoxytrityl group after each coupling cycle was accomplished by the action of 2% TFA in acetonitrile (or its mixtures with $CHCl_3$ and CH_2Cl_2) at 20°C. The preliminary analysis have revealed that in these conditions the detritylation was complete in 5-8 min, and the treatment did not cause any detectable depurination of protected derivatives of deoxyadenosine and deoxyguanosine [9]. The unreacted 5'-hydroxyl groups on polymers were masked before proceeding to the next step of the cycle by the treatment with acetic anhydride in the presence of MeIm. It was found that in solution the reaction is complete in 1 min with the use of 5-10% solutions of acetic anhydride and MeIm in different solvents ($CHCl_3$, CH_2Cl_2, acetonitrile, pyridine and there mixtures). Under these conditions, the acetilation reaction on solid supports is over within 2-5 min[9]. After the coupling reaction, the support was treated with dioxane-H_2O-DBU (7:2.5:0.5) to hydrolyze any N-base adducts that formed during the condensation. The coupling yields were determined by measuring the trityl cation absorbance spectrophotometrically and were found to be in the range of 75-90% with the use of 4-8 nucleotide-long blocks. The overall yields of polynucleotides (V) and (VI) and the synthesis schemes are shown in Table 1.

Deprotection and purification

The removal of the protecting groups from the final polynucleotides after completion of the synthesis was carried out in three stages without intermediate purification. (i) The substance was treated with the oximate solution to remove chloro-

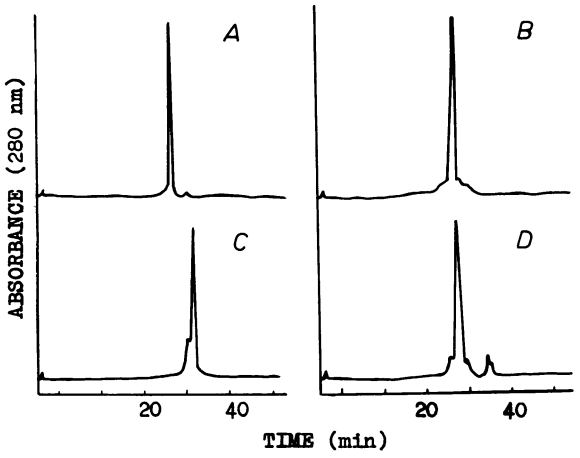


FIGURE 3. The HPLC analysis on Zorbax C-8 column of the purified synthetic 24-mer (II)(A), 32-mer (IV)(B), 32-mer (V)(C) and 42-mer (VI)(D).

phenyl protecting groups from internucleotide phosphates [17]. The use of oximate reagent enables to provide minimum unwanted cleavage of internucleotide bonds that is very actual for long oligonucleotides. (ii) Then the oligonucleotide was treated with concentrated ammonia to remove acyl protecting groups. (iii) The terminal 5'-dimethoxytrityl group was removed by the action of 80% acetic acid.

The deprotected polynucleotides were isolated by preparative

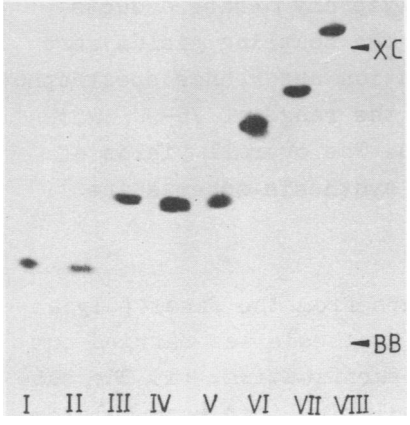


FIGURE 4. Analysis of the synthetic oligonucleotides (I) to (VIII), after labelling their 5'-termini, by electrophoresis on 10% polyacrylamide gel containing 7 M urea.

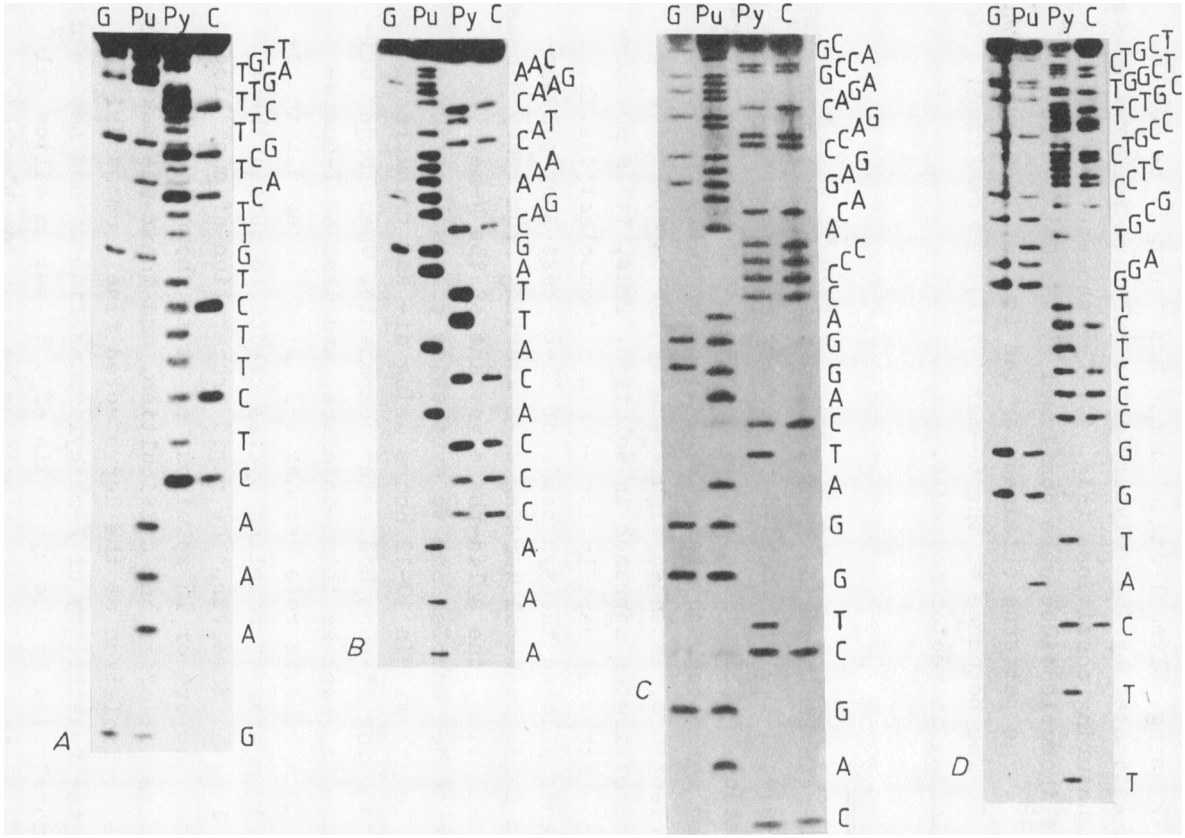


FIGURE 5. Sequence analysis of 5'-^{32}P-labelled polynucleotides: 32-mer (III)(A), 32-mer (V)(B), 42-mer (VI)(C) and 47-mer (VII)(D).

electrophoresis on denaturing polyacrylamide gels. After recovering from the gel and desalting, each oligomer was subjected to a reversed phase HPLC on Zorbax C-8 column (Fig. 1 and 3). Aliquots of the products were labelled at the 5'-end with [γ-^{32}P]ATP using polynucleotide kinase and found to be 80-90% pure as estimated by 10% polyacrylamide gel electrophoresis under denaturing conditions (Fig. 4). The sequences of ^{32}P-labelled oligonucleotides were confirmed by the Maxam-Gilbert method [18]. The patterns obtained for the four of them are shown in Fig. 5. The sequence of the 62-mer (VIII) was confirmed after cloning in vector molecule.

Conclusion

The N-methylimidazolide phosphotriester method has substantially reduced the time needed to perform each internucleotide condensation. Application of this method, along with the reversed phase chromatography for purification of fully protected oligonucleotides, extended significantly the capabilities of the phosphotriester approach in solution. Thus, it is now possible to synthesize rapidly long-chain oligonucleotides.These refinements allowed the synthesis of 30-60 base-long polynucleotides during several working days starting from ready blocks. At the same time, the solid-phase variant of the N-methylimidazolide method enables one to synthesize polynucleotides of similar length within 10-12 hours using di- and trinucleotides as the incoming 3'-phosphodiester components. When longer oligonucleotides, such as tetra-octamers, are used as P-components, the synthesis rate becomes 2-3 times faster due to reduction in number of coupling cycles needed to obtain the defined length. Moreover, the rapid preparation of such blocks on polymer support or in solution, as it is shown in this paper, does not offer any difficulties, and their use makes much easier isolation of the deprotected final compounds. Recently, the feasibility of N-methylimidazolide method has been also demonstrated by the synthesis of a 51-mer on silica gel support with the use of dinucleotides as starting blocks [19].

EXPERIMENTAL SECTION

Materials and general methods not mentioned below were as described previously [5,9]. The solvents were of the highest purity commercially available and were stored over molecular sieves. 5'-end labelling, chemical cleavage and gel electrophoresis during oligonucleotide sequence analysis were performed essentially as described [18].

Synthesis of fully protected oligonucleotides in solution

Preparation of nucleotide monomers and internucleotide condensations were performed essentially as it was described previously [5,9]. The protected mononucleotides (X) were purified before use by silica gel column chromatography[20]. Fully blocked 2-4-mers were also isolated by a short column silica gel chromatography using a linear gradient of methanol in chloroform (0-6%), whereas longer oligonucleotides (6-10-mers) were isolated by silica gel HPLC on Lichrosorb Si column (2.5 x 25 cm) in the same solvent. Fractions containing the desired product were pooled, evaporated in vacuo and precipitated from chloroform solution by dropwise addition into hexane. After centrifugation, the precipitate obtained was dried in vacuo.

The isolation of protected oligonucleotides starting from 14-mers was performed by reversed phase chromatography. After a short column silica gel chromatography (or without this step) oligonucleotide (500-1500 A_{280} units) was dissolved in 50-100 ml of starting buffer at 50°C (or in minimum volume of dioxane) and applied to a Nucleosil 30 C_{18} Macherey-Nagel column (1x25 cm) or onto a column with RP-2 silica gel (particals 40-60 μ) obtained from Kiezelgel 60 (Merk)[12]. Column was eluted at flow rate 2-4 ml/min with a gradient of 45-75% acetonitrile in 0.1 M triethylammonium acetate (pH 8.0) containing 10-20% dioxane, or with a gradient of dioxane (60-80%) in the same buffer. Oligonucleotide-containing fractions were combined and extracted with chloroform. The organic phase was evaporated, the residue was washed with ether and dried in vacuo.

The removing dimethoxytrityl group from the fully protected oligonucleotides was performed by the treatment of compounds with 2% solution of TSA in chloroform-methanol (7:3 , v/v) at 0°C for 2-3 min, or by the action of 2% TFA in chloroform-metha-

nol (9:1, or 19:1, v/v) at 20°C for 1-2 min. To deblock cyanoethyl group, an oligonucleotide was treated with 2 M solution of triethylamine in acetonitrile (20 ml/mmole) at room temperature for 0.5-1 h. After evaporation, the residue was precipitated from chloroform solution by addition into hexane-ether (2:1), centrifugated and dried in vacuo.

Functionalisation of supports

Silica gel and glass supports. Waters HPLC-grade silica (Porasil C, particle size 37-75 μ) was derivatized to create NH_2-groups as described by Chow et al.[21]. 1 g of this silica gel, or glass beads (CPG/long chain alkylamine pore glass, Pierce, 0.1 mmole amino groups per g) was mixed with succinic anhydride (0.5 g) and MeIm (425 μl) in dry pyridine (5 ml). The mixture was allowed to stand at room temperature for 10-15 h. After filtration, the support was washed with aqueous pyridine, dry pyridine, diethyl ether and dried in vacuo. Then 0.3 mmole of one of the four 5'-dimethoxytrityl-2'-deoxynucleoside in 5 ml of dry pyridine, 0.3 mmole of TPSCl and 0.9 mmole of MeIm were added to the support. After 1 h, 0.2 ml of ethanol was added, and the reaction mixture was allowed to stand additional 15 min. The support was sequentially washed with acetonitrile, chloroform, ether and dried in vacuo. Loading of the first nucleoside was found to be in the range of 50-100 μmole/g as determined by A_{498} of dimethoxytrityl cation released.

Polystyrene support. 5 g of chloromethyl polystyrene (1% cross-linked by divinylbenzene, 1.25 mmole/g, Bio-Rad) was swollen by addition of 100 ml of 0.1 M NaOH solution in dioxane -H_2O (4:1) containing 2-mercaptoethanol (2 ml), and the mixture was shaken at 80°C for 5-6 h was filtered. The resin was washed with aqueous dioxane, methanol, dioxane. The resulting polymer was suspended in 70 ml of dioxane, then 5 ml of acetic acid and 100 mg of $Na_2WO_4 \cdot 2H_2O$ dissolved in H_2O (10 ml) were added. After heating to 80°C, 30% H_2O_2 (15 ml) was added dropwise under stirring during 2 h. The mixture was allowed to stand at the same temperature for additional 0.5 h. After filtration, the resin was washed with 80% dioxane, water, dioxane, methanol and dried in vacuo. The support (1.2 mmole S/g) was then reacted with 0.5 equivalents of a 5'-O-dimethoxy-

trityl-2'-deoxynucleoside-3'-chlorophenyl phosphate in pyridine (10 ml) in the presence of TPSCl (1 equiv.) and MeIm (2 equiv.) for 0.5 h and then washed with dichloromethane and pyridine. The unreacted OH-groups on the polymer were capped by the action of phenylisocyanate-pyridine (1:9) as described [20]. The amount of nucleotide attached to the polymer was estimated by the quantitative analysis of dimethoxytrityl group and nucleotide liberated from the resin. Typically nucleotide content was 0.4 mmole/g.

Oligonucleotide assembly on polymer supports

The various chemical operations performed for the addition of one coupling unit to the polymer supports, the amount of reagents and the reaction times are listed in Table 2. The coupling yields were estimated by the spectroscopic analysis of the dimethoxytrityl function liberated from the resin [22].

Synthesis of short oligonucleotides with 3'-terminal phosphodiester group was carried out starting from 400 mg of functionalized polystyrene carrier (XIV) in a vessel similar to that described by Ito et al.[23]. Cycles of wash and nucleotide addition were continued until the required length was reached. After the final coupling reaction the support was washed with chloroform, methanol, ether and dried. The removal of oligomer was performed by the action of 30% solution of triethylamine in dioxane for 2-3 h at 20°C (1 ml/100 mg of polymer). Filtrate and washings were avaporated, and the desired product was isolated by reversed phase chromatography as shown in Fig. 2.

Synthesis of polynucleotides on functionalized silica gel and glass supports (100-150 mg) was carried out in the glass column (3 x 50 mm). The column was initially washed with acetonitrile, and the capping reagent was passed through column during 5 min. After the washing, the first reaction cycle was carried out as outlined in Table 2. The synthesis of 32-mer (V) was accomplished on the glass support with average coupling yield per cycle 85%. The 42-mer (VI) was synthesized on the silica gel support with average coupling yield per cycle 75%.

Removal of protecting groups and isolation of polynucleotides

The final oligonucleotides obtained in solution, or the support containing the desired oligonucleotide, was treated with

0.5 M solution of 1,1,3,3-tetramethylguanidinium-2-pyridinealdoximate in dioxane-water(1:1) for 16-18 h at 37°C, and the mixture was evaporated to dryness. The residue was dissolved in concentrated ammonia solution (10 ml). After 5-7 h standing at 50°C, the solution was evaporated. The residue was treated at room temperature with 80% acetic acid for 15 min. The solution was diluted with water and extracted with diethyl ether. The aqueous phase was evaporated to dryness, desalted and taken up in 7M urea.

The isolation of deprotected oligonucleotides was accomplished by preparative electrophoresis on 3 mm thick denaturing 8-10% polyacrylamide gels as described [5,11]. Then oligomers were subjected to reversed phase HPLC on Zorbax C-8 column (4x250 mm) (Fig. 1 and 3). After ^{32}P-labelling, the purity of polynucleotides was proved by gel electrophoresis (Fig. 4),and their sequences were confirmed by the Maxam-Gilbert method [18](Fig. 5).

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15. Besides the support described above, we have examined the carrier on the base of polystyrene modified with $HO-CH_2CH_2-SO_2-CH_2-CO-NHCH_2$-groups. The experiments revealed that this support is also very useful for the synthesis of oligonucleotide blocks with 3'-phosphodiester groups. Details to be published elsewhere.
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